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(54) Title: FACTORS HAVING PRION-BINDING ACTIVITY IN SERUM AND PLASMA AND AGENTS TO DETECT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHITIS

(57) Abstract: Disclosed are methods and tools for the concentration and detection as well as quantification of pathological prion proteins as well as agents to be used in said detection and/or in the prevention or treatment of prion diseases. Said agents are factors with prion binding activities found in blood serum and blood plasma.

WO 01/23425 A1

FACTORS HAVING PRION BINDING ACTIVITY IN SERUM AND PLASMA AND AGENTS TO DETECT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHITIS

Cross Reference to Related Application

5   This application claims the priority of US patent application 09/407,667, filed September 28, 1999, the disclosure of which is incorporated herein by reference in its entirety.

10   ### Field of the Invention

The present invention concerns a method and agents to detect transmissible spongiform encephalopathies as well as agents for the prevention and
15   treatment of respective infections.

Background Art

According to all available evidence, the
20   agents causing transmissible spongiform encephalopathies, termed prions, are devoid of informational nucleic acids and consist of an "infectious" protein (termed PrP^{Sc}) capable of converting a normal host protein called PrP^C into a likeness of themselves. The only organ system in
25   which histopathological damage and its clinical sequelae can be demonstrated as a consequence of infection with prions is the nervous system (Brandner et al., 1996). This consideration applies to both the human transmissible spongiform encephalopathies, such as
30   Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker Syndrome, Kuru and fatal familial insomnia, and all known prion encephalopathies of animals (Weber and Aguzzi, 1997). The latter comprise scrapie in sheep, bovine spongiform encephalopathy, and chronic wasting diseases
35   of mule, deer and exotic ungulates (Weissmann and Aguzzi, 1997).

However, there is no doubt that prions, herewith operationally defined as the infectious agents causing transmissible spongiform encephalopathies, can colonize organs other than the central and peripheral
5 nervous system, and can be demonstrated in extracerebral compartments (Aguzzi et al., 1997). The problem of which organ systems can harbour infectivity is further complicated by the existence of prion strains. Just like strains of conventional viruses, prions can come in
10 various different flavors, each one of which has its specific preferences with regard to the host range which is infectible and also to the type of cells in which it replicates (Aguzzi, 1998). One paradoxical situation, which is of immediate relevance to the question of blood
15 safety, is exemplified by the radically different organ tropism of the BSE agent in cows and in humans. BSE prions seem to be largely confined to the neural compartment of cows, even after oral exposure (Wells et al., 1998). A very accurate study of the pathogenesis of
20 experimental BSE in cows upon feeding 100 grams of infected brain has disclosed that there is only a short and transient period during which infectivity can be demonstrated in the terminal ileum (Wells et al., 1998). At later time points, BSE prions can only be shown in
25 brain, spinal cord, and dorsal root ganglia. The exact localization of BSE in the terminal ileum is not known. It is being discussed whether infectivity resides in Peyer's patches or in the neural compartment which comprises the Plexus submucosus Meissner and the Plexus
30 myentericus Auerbach. There is a great body of circumstantial evidence that BSE prions can provoke new variant Creutzfeldt-Jakob Disease (nvCJD) (Bruce et al., 1997; Chazot et al., 1996; Hill et al., 1997; Will et al., 1996), but no absolutely final evidence has been
35 produced. For the purpose of the following discussion we will regard the evidence that BSE and new variant Creutzfeldt-Jakob Disease caused by the same agent as

sufficiently verified (Aguzzi and Weissmann, 1996). Upon passage into humans, and consecutive progression to manifest nvCJD, prions experience a dramatic shift in their organotropism. Instead of remaining confined mainly
5   to neural structures, they can be detected in many organs belonging to the immune system, including most notably tonsils, spleen, and as recently demonstrated, the appendix (Hilton et al., 1998). It is, therefore, unavoidable to conclude that the tropism of the
10 infectious agent for various structures depends on both the strains of prions in question (and therefore it is in part autonomous to its carrier) and on the species in which prion disease manifests itself (Aguzzi and Weissmann, 1998).

15   These considerations are not only of academic interest. In fact, the transmissibility of the agent by iatrogenic manipulations (i.e. blood transfusions, organ transplants, etc.) is crucially affected by such parameters.
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          Horizontal Transmissibility of Human Prions:
          Prion diseases of humans are undoubtedly transmissible. However, transmission is achieved only under particular circumstances. One could say that in
25 this respect prion diseases fulfill the characteristics of transmissibility delineated by Semmelweiss for puerperal fever: these affections are infectious but not contagious. Direct transmissions of brain-derived material from a patient suffering from Creutzfeldt-Jakob
30 disease to other persons have documentedly resulted in transmission of disease. A particularly tragic case occurred in the early seventies in Zurich, when electrodes used for cortical recordings from Creutzfeldt-Jakob patients were sterilized (formaldehyde and alcohol)
35 and used in additional patients. Disease was transmitted to the very young recipients (Bernoulli et al., 1977).

Also, transplantation of cornea has most likely resulted
in transmission of disease (Duffy et al., 1974).

Despite these tragic dimensions, cases of
iatrogenic transmission of CJD via neurosurgical
5   procedures have remained rather rare. This is not totally
understood, given that the frequency of subclinical CJD
must be much higher than that of manifest disease, and
that most neurosurgical instruments are not sterilized in
a way that would reliably inactivate prions. Therefore,
10   the quite rare nature of iatrogenic transmission is
likely to indicate that host factors, in addition to the
virulence of prions, may affect the probability that
infection takes place. This notion is strengthened by the
epidemiology of iatrogenic CJD (iCJD) upon transmission
15   of contaminated dura mater. It has been estimated that
several thousands patients, predominantly in Japan, may
have been exposed to the CJD agent via preparations of
cadaveric dura mater which had been contaminated with
prions. However, it appears that less then 2% of those
20   exposed have developed disease so far. While we can
rejoice about this low efficiency in the "take" of
infectivity, we do not fully understand the biological
basis for the apparent protection enjoyed by most
subjects exposed to CJD prions. The largest problem with
25   iatrogenic transmission has occurred as result of
administration of pituitary hormones of cadaveric origin
(Gibbs et al., 1985). Preparations of growth hormone and
of gonadotropins contaminated with human prions have
caused the death of more then 80 persons, predominantly
30   children. Due to the long latency that can be expected
when the agent is introduced into extracerebral sites,
such as via intramuscular injection, it must be assumed
that further cases from this procedure, which has been
stopped more than a decade ago, will arise in the future.
35   Besides its tragic human dimension and the
harm that it has cost to the patients and to their
physicians, the pituitary hormone disaster needs to be

understood in detail, because the anterior lobe of the
pituitary gland is not a part of the central nervous
system. Therefore, these events may serve as a paradigm
for transmission of prions via contaminated extracerebral

5   tissue that does not belong to the canonical sites of
replication of prions. The observation that latency after
intracerebral contamination is much shorter than latency
after peripheral infection is in good agreement with
experimental data from various animal models, and suggest

10   that a rather lengthy phase of extracerebral events
(which may include replication of the agent, and invasion
of specific extraneuronal systems) may be a precondition
to prion neuroinvasion (Aguzzi, 1997).

15   Factors influencing the neurotropism of
prions:
          There is good reason to suspect that
neuroinvasive processes in the course of prion infections
are very tightly controlled. Perhaps the best argument in

20   this respect derives from the observation that the
incubation times of experimental animals inoculated
intraperitoneally with scrapie prions are extremely
reproducible. Upon inoculation with a known amount of
standard inoculum, the experience in various laboratories

25   has been that latencies between inoculation and first
clinical symptoms display standard variations in the
order of only a few percent points (Klein et al., 1997).
If prion neuroinvasion were a totally random process, one
would expect a large variability in the incubation times,

30   which would depend on processes governed by chance.
However, if some rate-limiting processes control
neuroinvasion, these may be responsible for the
remarkable precision of the incubation times. Indeed, we
very much hope that this interpretation is correct

35   because if such processes exist they might be amenable to
manipulation, which in turn may represent a post-exposure
strategy to prevent overt prion disease. Indeed, various

mechanisms have been explored by which neuroinvasions may be accomplished.

A first phase or neuroinvasion seems to be widespread colonization of the immune system. This

5 colonization can be visualized by homogenizing spleen, lymph nodes, tonsils, and also appendix, and injecting the homogenates into suitable experimental animals. The dilution of the homogenates at which 50% of the experimental animals become sick, contains one ID50 of

10 the infectious agent in each inoculum.

The second phase of neuroinvasion seems to be dependent upon a compartment which cannot be replaced by adoptive bone marrow transfer (Blättler et al., 1997) and which may be represented by the peripheral nervous system

15 and/or the follicular dendritic cells resistant to germinal center of secondary lymphatic organs. It appears that this second compartment necessitates the expression of normal prion protein in order to support neuroinvasion (Blättler et al., 1997).

20   Neuroinvasion is dependent on a functional immune system, and immunodeficient mice do not develop disease after inoculation with a moderate dose of the agent (Fraser et al., 1996; Kitamoto et al., 1991; Lasmezas et al., 1996; O'Rourke et al., 1994). One

25 crucial component of the immune system necessary for neuroinvasion has been traced to the physical presence of terminally mature B-lymphocytes . To date, it is not clear whether B cells are required because they bind physically prions and carry them to sites of

30 neuroinvasion, or whether B cells produce factors, or induce processes, which are indirectly responsible for facilitating neuroinvasion (Klein et al., 1997). Given the requirement for B-lymphocytes secreting lymphotoxin for the maturation of follicular dendritic cells, and the

35 fact that follicular dendritic cells accumulate large amounts of scrapie prions in experimental situations, it is tempting to speculate that the main function of B-

lymphocytes in the aforementioned process consists in
allowing FDCs to mature.

        The cellular and molecular basis of prion
5   neuroinvasion:
            Following experimental inoculation of mice
with prions at peripheral sites, there is typically a
prolonged, clinically silent replication phase of the
infectious agent within the lymphoreticular system (LRS).
10 This occurs prior to detectable neuroinvasion by prions
and the subsequent occurrence of neurological symptoms.
During this preclinical latency period, prions may
replicate to high titers within lymphoreticular tissues.
Elucidating the cell types in which prions replicate
15 within the peripheral lymphoid tissue and - crucially -
how prions are transported to the central nervous system
(CNS) is of great interest and clinical importance.
Despite considerable evidence implicating the role of the
immune system in peripheral prion pathogenesis, there
20 have been few studies on the identity of the cells
involved in this process. It has been shown many years
ago that whole-body irradiation of mice with gamma rays
fails to influence prion pathogenesis or incubation time
of scrapie. This has been taken as an argument against
25 significant involvement of proliferating cells in the
lymphoreticular phase of prion propagation. Instead,
follicular dendritic cells (FDC) have been considered as
the prime cell type for prion replication within lymphoid
tissue since PrP^{Sc} accumulates in the follicular
30 dendritic network of scrapie infected wild-type and nude
mice (Kitamoto et al., 1991). In addition, severe
combined immuno deficient mice (SCID), which lack mature
B- and T-cells, and which do not appear to have
functional FDCs, are highly resistant to scrapie after
35 intraperitoneal inoculation and fail to replicate prions
in the spleen (Fraser et al., 1996; Kitamoto et al.,
1991; Lasmezas et al., 1996; O'Rourke et al., 1994).

Interestingly, bone-marrow reconstitution of SCID mice
with wild-type spleen cells restores full susceptibility
to scrapie after peripheral infection (Fraser et al.,
1996; Klein et al., 1998). These findings suggest that an
5   intact, or at least partially functional, immune system
comprising lymphocytes and FDC is required for efficient
transfer of prions from the site of peripheral infection
to the CNS.

The time course for the development of
10   scrapie disease following intracerebral or
intraperitoneal inoculation is highly reproducible and is
primarily dependent on the dose of the inoculum.
Therefore, neuroinvasion by prions migrating from
peripheral lymphoid tissue may depend on tightly
15   controlled, rate-limiting reactions. In order to identify
such rate-limiting steps during prion neuroinvasion, PrPC
deficient mice bearing PrP-overexpressing cerebral
neurografts were infected intraperitoneally (i.p.). No
disease was observed in the grafts, suggesting that
20   neuroinvasion depends on PrP expression in extracerebral
sites. This was further underlined by reconstitution of
the lymphoid system with PrPC expressing cells, which
restores infectivity in the lymphoid tissue, but still
fails to transport prions to the nervous system.
25   As prions can be detected in lymphoreticular
tissues, an understanding of the peripheral pathogenesis
is of immediate importance in assessing risks of
iatrogenic transmission of human BSE via exposure to
blood or tissues from preclinical cases, and possibly
30   from contaminated surgical instruments, or even blood and
blood products. Additionally, such advances might pave
the way for the development of sensitive diagnostic tests
and the means to block prion neuroinvasion. Why is
contamination of the blood supply with prions an
35   important issue? The main problem is new variant CJD. For
one thing, we by far do not know as much about the
epidemiology and iatrogenic transmissibility of this new

disease as we do for sporadic CJD (sCJD). What is most
unsettling, the distribution of preclinical disease in
Great Britain and possibly in other countries is very
obscure, and the little knowledge that is being gathered

5   is far from reassuring (Will et al., 1999). Moreover,
there is all reason to believe that nvCJD may be much
more "lymphoinvasive" than its sporadic counterpart. In
particular, nvCJD prions can be easily detected in
lymphatic organs such as tonsils and appendix (Hill et

10 al., 1999; Hill et al., 1997; Hilton et al., 1998), a
fact that was previously demonstrated to be true for
scrapie (Schreuder et al., 1997; Schreuder et al., 1998;
Vankeulen et al., 1996), but not for sCJD prions. While
all available evidence points to follicular dendritic

15 cells as the prion reservoir in lymphatic organs, splenic
lymphocytes of experimentally inoculated mice can be
infected with prions (Raeber et al., 1999). Although
prion infectivity of circulating lymphocytes appear to be
at least two logs lower than that detected in splenic

20 lymphocytes (Raeber et al., 1999), the possibility that
circulating lymphocytes may be in equilibrium with their
splenic siblings call for cautionary measures. The nature
of the latter is still matter of controversy and debate:
leukodepletion has been advocated, but at present there

25 is no certainty about its efficacy, and even whether the
presently available technologies for leukoreduction are
necessary and/or sufficient for decreasing the threat to
blood supply that derives from nvCJD. In addition, it has
to be taken into account that, even if blood prion

30 infectivity were to be originally contained in
lymphocytes in vivo, lysis of cells may lead to
contamination of non-particulate fractions and, in the
absence of appropriate measures of removal, of stable
blood products.

35   The second consideration applies so secondary
prophylaxis. Given the very large numbers of infectious
BSE material that has entered the human food chain, it is

possible that many individuals harbor preclinical nvCJD.
It is imperative and urgent to develop strategies that
will help control spread of the agent and that will
hopefully prevent the clinical outbreak of symptoms in
these persons. Possible targets for the interference with
neuroinvasion are rate-limiting processes that control
prion replication within the infected individual. In
light of the knowledge discussed above, treatments that
target the neuro-immune interface of prion replication
and neuroinvasion (Aguzzi and Collinge, 1997) seem a
promising area for research aimed at post-exposure
prophylaxis.

Methods to detect prions and their
limitation:

In the age of real-time kinetic polymerase
chain reaction (PCR), we have become very spoiled with
respect to the detection thresholds which we demand from
assays geared at detecting viral contaminants in blood.
Consider the case of HIV: here the introduction of
quantitative PCR technologies has pushed the limit of
detection in blood and blood products down to quasi-
perfection. Even when PCR techniques have not proved that
useful, or have not yet met with such widespread
acceptance, ultrasensitive immunochemical methods, such
as time-resolved fluorescent ELISA, have progressed to a
degree of sophistication that is highly satisfactory for
most screening application. So why do we still have a
problem with prion detection in blood?

The most formidable problem derives from the
unique biology of the prion. According to more-or-less
accepted wisdom, infectious prions are likely to consist
solely of the PrP^{Sc} protein, which has exactly the same
amino acid structure as the normal cellular protein PrP^{C}.
A more noncommittal way of wording this fact would be to
state that PrP^{Sc} is the only known surrogate marker for
prion infectivity: this latter statement is likely to be

agreeable upon by both the proponents of the protein-only hypothesis and by those who still believe that the infectious agent is a virus.

The consequence of the fact mentioned above

5 for prion detection is obvious: if prion-specific nucleic acids do not exist, any PCR-based screening assay to detect said nucleic acid will not be an option. Therefore, we are left with immunochemical assays. Besides being less sensitive than PCR by several orders

10 of magnitude, these are also fraught with a series of prion-specific problems. The biggest trouble, again, derives directly from the peculiar biology of TSE agents. As explained above, PrP^{Sc} possesses the same chemical composition as PrP^{C}, and the latter is a membrane-bound

15 protein that is normally found in many cell types of healthy individuals including white blood cells (Aguzzi and Weissmann, 1997). Although PrP^{C} and PrP^{Sc} differ in a number of physical properties, it appears to be extremely difficult to develop immunological reagents which

20 reliably differentiate between these two isoforms. Only one monoclonal antibody has been described to react with PrP^{Sc} but not with PrP^{C}, and its practical usefulness remains to be demonstrated since fourteen months after its publication no follow-up studies have appeared and

25 even the company which developed this reagent in the first place does not appear to use it in its in-house screening assay for BSE prions.

The hitherto best method for the detection of prions is by performing Western blot analysis with

30 homogenized brain tissue that has been digested with proteinase K (PK). The digestion is necessary since for Western blot analysis the secondary structure is broken up so that no difference is found any more between cellular prions (PrP^{C}) and pathological prions (PrP^{Sc}),

35 however, while PrP^{C} is readily digested by PK under specified conditions, PrP^{Sc} is only degraded to relatively large fragments called PrP^{27-30}.

It is also already known to concentrate
proteins by adsorbing them to so called magnetic beads
(MB) to which a specific antibody is bound. However, the
application of such a concentration method to PrP has

5 been assumed to be impossible due to the specific
features of prions.

Thus, still a great need exists to have a
sensitive method or test to detect small amounts of
prions not only for diagnosis but also for further

10 investigating the disease, as well as agents to perform
such tests.

Brief Description of the Invention

15 Hence it is one object of the present
invention to provide a method for the detection of the
pathological prion protein as PrP^{Sc} or PrP^{27-30},
respectively.

It is another object of the present invention

20 to provide a method for searching for prion interacting
agents.

Still another object of the present invention
are agents recognizing PrP^{Sc} and/or PrP^{27-30}.

Still another object of the present invention

25 are solid phase materials such as e.g. magnetic beads
carrying such agents and composition comprising same.

Still another object of the present invention
are compositions comprising such agents for purifying
body fluids and sterilization of surgical and diagnostic

30 instruments.

In the method of the present invention for
the concentration of PrP^{Sc} or digestion products thereof,
a body fluid, such as e.g. blood, urine, cerebrospinal
fluid etc., or fluidized organ, such as brain tissue,

35 lymph nodes, tonsils etc., is treated with a solid phase
material such as magnetic beads(MB) whereby at least part
of said material or beads, respectively, carries a prion

binding site. A preferred prion binding site is a factor
with prion binding activity (PrPB).

    The method works very well with a fluidized
organ, in particular homogenized tissue of central
5 nervous system, preferably homogenized brain tissue.

    In several cases the prion binding site can
only distinguish PrP^C and PrP^{Sc} in digested form. For
such cases, it is necessary to digest the fluid or
fluidized organ prior to the actual concentration step. A
10 suitable digestion is obtained by digestion with
proteinase K (PK), whereby it is important to inactivate
the proteinase K prior to the addition of the solid
phase, e.g. MBs.

    Preferred solid phase materials carrying PrPB
15 are prepared by coupling such materials with blood serum,
or blood plasma, such as fresh frozen plasma of mammals,
whereby an excess of protein is present during the
coupling procedure. Such factors are designated spPrPB
(s= serum and p= plasma) (see below). Even more preferred
20 solid materials carrying PrPB are prepared by coupling
purified plasminogen or fibrinogen to the beads(see
below).

    Very suitable solid materials are magnetic
beads since they can easily be treated with specific
25 components of interests and easily be collected by
applying a magnetic field.

    A further method of the present invention
concerns the detection (and optionally quantification) of
PrP^{Sc} or digestion products thereof, wherein PrP^{Sc} is
30 first concentrated as described above, optionally also
with previous digestion of the fluid or fluidized organ,
and then detected and optionally compared with a
standard. A suitable detection method is Western blot
analysis. Such test may furthermore be embodied by other
35 detection methods such as a microtiter plate format
immunoassay (e.g. ELISA assay), an immunoprecipitation

assay, a BIACORE assay, immunocytochemical assay,
histoblot assay etc.

Besides of the above mentioned methods, the
present invention also concerns factors with prion
5   binding activities such as sPrPBII, which is a prion
binding activity in fraction II of ammonium sulfate
precipitation of serum or pPrPBII which is a factor with
prion binding activity in fraction II of ammonium sulfate
precipitation of normal or fresh frozen plasma. Said
10 factors are of course subject matter of the present
invention in any form, such as in isolated form, or as
ingredient in a composition, e.g. in a fraction of
ammonium sulfate precipitation.

Said factors can be obtained by concentration
15 and/or isolation of PrPBs whereby serum or plasma is
subjected to fractionated ammonium sulfate precipitation
thus that a PrPB of interest is precipitated, preferably
in only one fraction. A further purification can be
obtained by the application of further protein isolation
20 methods.

The factors of the present invention are not
only suitable for the detection of prions, but they have
further applications in methods for the purification and
removal of pathological prion protein from body fluids
25 and organs, such as blood, urine, cerebrospinal fluid,
brain tissue, lymph nodes, tonsils etc., or for the
sterilization of surgical and/or diagnostic tools, basing
on the affinity of PrPB for the pathological prion
protein. They are furthermore tools for a therapy regimen
30 based on the modulation of production of PrPB for
preventing the spread of prions in the body. Especially
suitable in this respect is plasminogen, that is also
especially suitable for the purification of body fluids,
e.g. blood units. Such purification may e.g. be performed
35 by treating fluids with PrPBIp, in particular with
immobilized plasminogen or plasma fractions containing
same.

Also part of the present invention is a test
for the detection of pathological prion protein in body
fluids or organs such as blood, urine, cerebrospinal
fluid, brain tissue, lymph nodes, tonsils etc, that
5   utilizes the specific binding properties of PrPB to
pathological prion protein. Such test can be embodied as
a microtiter plate format immunoassay, e.g. ELISA assay,
an immunoprecipitation assay, a BIACORE assay,
immunogytochemical assay, histoblot assay etc.
10   Also the DNA sequences specific for
biosynthesis of PrPB are comprised by the present
invention as well as vectors able to express such DNA
sequences in suitable hosts.
          Furthermore comprised by the present
15   invention are: a method for purification of PrPB by using
PrP$^{27-30}$ as bait; monoclonal and polyclonal antibodies
produced in animals such as mice, rabbits, chicken etc.,
and directed against PrPB; single-chain Fv fragments and
other types of fragments of antibodies produced in
20   recombinant phages or in other recombinant systems, and
directed against PrPB; a test predictive of
susceptibility to prion diseases based on polymorphisms
of PrPB, or on variations in the strength and pattern of
production of PrPB; a transgenic animal, e.g. mouse that
25   overproduction PrPB in brain, lymph nodes, or other
organs, to be used in a bioassay for prions; a knockout
animal, in particular a mouse, which is devoid of PrPB,
to be used in a bioassay for prions; a production method
of PrPB by expressing a DNA sequence specific for the
30   biosynthesis of PrPB in a suitable host cell, such as
bacteria, yeast, fungi, or eukaryotic cells, and by
purification of PrPB from the aforementioned organisms; a
use of natural or synthetic, preferably purified PrPB as
a medicament for therapeutical applications in humans and
35   animals; a vaccination of organisms with natural or
synthetic PrPB, in particular plasminogen; a diagnostic

assay for human and/or animal diseases resulting from
abnormal production and/or metabolism of PrPB.

5   Brief Description of the Drawings
            Figure 1 is a scheme showing the IAP method.
            Figure 2 shows Western Blots and IAP
experiments of dilution experiments, whereby lanes 1 to 6
and 10 represent usual Western Blots and lanes 7 to 9 and
10 11 to 13 represent immuno affinity purification (IAP).
            Figure 3 is a scheme showing the prion
affinity assay (PAA) method.
            Figure 4 represents Western Blots showing
positive and begative controls of the PAA.
15   Figure 5 shows the observation that beads
coated with sheep anti mouse IgG Abs by DYNAL bind PrPSc
but not PrP27-30. Upon preincubation with normal mouse
serum PrP27-30 is also bound.
            Figure 6 represents Western Blots showing the
20 results with serum proteins that are coupled to beads.
The * means that the coupling was performed in the
presence of an excess of proteins.
            Figure 7 shows the effect of the addition of
PK-treated brain homogenate to the assay.
25   Figure 8 represents Western Blots showing the
results with PrP-deficient material.
            Figure 9 represents Western Blots showing PAA
of ammonium sulfate precipitates.
            Figure 10 represents Western Blots showing
30 PAA of ammonium sulfate precipitates that are not
covalently crosslinked to the beads.
            Figure 11 shows the result of the PAA of the
58 fractions of human plasma that were obtained by
chromatography and differential precipitation and tested
35 for binding activity.
            Figure 12 represents Western Blots showing
the results with purified plasminogen and fibrinogen.

Figure 13 represents Western Blots showing the calcium dependency of the binding activity of plasminogen and fibrinogen.

Figure 14 represents Western Blots showing the dependency of the binding activity of plasminogen on the native state of the proteins.

Figure 15 represents Western Blots showing PAA of plasminogen that is not covalently crosslinked to the beads.

Figure 16 shows the concepts of the bioassay.

Figure 17 shows the results of the bioassay.

Detailed Description of the Invention

As already mentioned above, there is a great need for a detection method for low concentrations of PrP^{Sc} that can be used as a diagnostic test for transmissible spongiform encephalopathies (TSEs).

There are basically three diagnostic principles for TSEs: histopathological detection of the typical spongiform changes in the CNS, detection of the scrapie-specific isoform of the prion protein, and the bioassay that detects infectivity. All these methods have limitations: histopathology is not useful for preclinical diagnosis since the structural changes appear late in the incubation period. Detection of the scrapie the Western specific is form of prion protein is more sensitive but still much less sensitive than the bioassay. The bioassay can, in principle, detect as little as 1 infectious unit but can last months or even years.

The hitherto used Western blot technique is based on the partial protease resistance of PrP^{Sc} that allows to distinguish between PrP^{C} and PrP^{Sc}. After protease treatment, PrP^{27-30} – the protease resistant core of PrP^{Sc} – can be detected but not PrP^{C} which is completely digested.

Although due to the "stickiness" of prions it
was generally assumed that immuno affinity purification
(IAP) cannot be applied, it has now been found that
concentration can be achieved by applying magnetic beads
5   (MB) carrying a prion binding site, preferably a factor
with prion protein binding activity (PrPB).

Thus, because the sensitivity of detection of
absolute amounts of PrP^{27-30} is a function of antibody
affinity, and cannot be easily increased for each given
10   antibody, in the scope of the present invention, despite
of the hitherto assumed problems, first an "immuno
affinity purification" (IAP) assay has been developed,
using antibodies covalently crosslinked to solid phase
material, e.g. magnetic beads. Because the monoclonal
15   antibody (6H4 purchased at Prionics, Zurich, Switzerland,
described in Korth et al., 1997), originally used for the
development of the IAP, is not able to distinguish
between PrP^C and PrP^{Sc} (it binds both undigested forms as
well as digested PrP^{Sc}, i.e. PrP^{27-30}), it is necessary
20   to perform Proteinase K digestion prior to the IAP (see
Fig.1).

For the development of the present IAP
method, the following model system was used: Two tests
were performed to determine the efficiency of the method.
25   On the one hand, small amounts of a scrapie-infected
mouse brain homogenate were diluted with water and then
subjected to the PrP^{Sc} concentration method. On the other
hand, small amounts of a scrapie-infected brain
homogenate was diluted with brain homogenate of non-
30   infected mice in order to simulate a real situation in
which a brain homogenate contains low amounts of PrP^{Sc}
(see Fig.2).

In Figure 2, lanes 1 to 6 and 10 represent
usual Western Blots and lanes 7 to 9 and 11 to 13
35   represent immuno affinity purification (IAP). PrnP% is
material from PrP deficient mice. MB are of course only
used for IAP whereby 6H4 refers to MB coupled with 6H4

antibodies and – refers to uncoupled MBs. PRP^C refers to
brain homogenate of non-infected mice and PrP^{Sc} refers to
brain homogenate of scrapie-infected mice. PK refers to
Proteinase K digestion whereby – refers to non digestion

5   and + to digested homogenate. The same abbreviations are
used for the following figures.

For prion analysis in homogenate, in
particular of brain tissue, it is important to use in a
first homogenation step low concentration of ionic

10 detergent, followed by low speed centrifugation,
preferably 500 g 30 minutes, 4°C applied twice. For
following steps high concentration of non-ionic detergent
is used and a protein concentration of the homogenate of
at most 5 mg/ml.

15 Conditions for the proteinase K digestion are
preferably 50 µg/ml PK, 37°C and at least half an hour.

Suitable incubation conditions for the beads
with homogenate are e.g. about 1.5 hours at room
temperature, whereby for low concentrations longer

20 incubation times might be preferable.

The concentration step in said first attempt
was carried out by adding to digested homogenate magnetic
beads (MB) carrying said 6H4.

If a digestion step is needed, it has to be

25 performed prior to the concentration step, whereby the
digestion, usually by proteinase K, has to be stopped
prior to the concentration step by deactivating the
proteinase e.g. with phenyl methyl sulfonyl fluoride or
another agent known to the skilled person.

30 By applying the method of the present
invention for e.g. brain tissue homogenate, PrP$^{27-30}$ can
be concentrated up to amounts detectable by Western blot
analysis from tissue comprising much less pathological
prion protein than needed for the hitherto known tests.

35 Using largely the same procedure, the above
described method can also be applied as prion affinity
assay (PAA) by exchanging the monoclonal antibody 6H4 by

other substances to be examined, for example in order to
find a binding partner for PrP^{Sc} (see Fig.3).

As a positive control of this assay 6H4 (see
5   Figure 4, lanes 1-3) is used and as a negative control
mouse IgG or mouse albumin (see Figure 4, lanes 4-9).
In order to investigate whether a given mouse
serum containes IgG that specifically recognize PrP^{Sc}
magnetic beads that are already coated by the company
10 DYNAL with sheep antibodies directed against mouse IgGs
were used after preincubation with mouse serum. These
beads – used without preincubation – were the first
negative control (see Figure 5, lanes 1-2). As a second
negative control these beads preincubated with normal
15 mouse serum were used in order to show that IgGs from
normal mouse serum do not bind to any form of PrP (see
Figure 5, lanes 3-4). Surprisingly the beads alone showed
an affinity to PrP^{Sc} but not to PrP$^{27-30}$. Upon
preincubation with normal mouse serum also PrP$^{27-30}$ is
20 bound. Therefore it was hypothised that the sheep
antibodies from DYNAL recognize a molecule that is
associated with PrP^{Sc} but digested away after PK-
treatment. As PrP$^{27-30}$ is bound upon preincubation with
normal mouse serum, this serum might contain the molecule
25 with affinity to PrP^{Sc}.
The beads coupled to total mouse serum
proteins did not show any affinity to any form of PrP.
However, if the coupling of the total serum was performed
in the presence of an excess of protein the beads showed
30 the same binding to PrP$^{27-30}$ as the monoclonal antibody 6H4
(see Figure 6, lanes 4-6) whereas the beads that were
coupled in the presence of an excess of albumine still
did not show any affinity to any form of PrP (see Figure
6, lanes 1-3). Though it was not possible to measure any
35 difference of the coupling efficency of the two
conditions it might be that offering an excess of
proteins causes a sponge on the surface of the beads that

binds PrP^{27-30}. We also checkled whether PK-treated brain
homogenate might enhance the binding as in the case of
bound PrP^{27-30} total PK-digested brain homogenate is
present: the addition of PK-digested brain homogenate
5   from wild-type C57BL/6 mice or $Prnp^{o/o}$ mice allowed to
bind PrP^{Sc} in addition to PrP^{27-30} (see Figure 7, lanes 1-
3); the addition of inactive PK had no influence on the
binding activity (see Figure 7, lanes 7-9). If coupled in
the presence of an excess the activity of binding PrP^{27-30}
10   was also found in the serum of man, sheep, cow and in the
serum of terminally scrapie-sick C57BL/6 mice (data not
shown).

            Apart from an artefact it might well be that
serum of several species contains activities
15   (collectively termed PrP^{B}) that interact specifically
with the pathogenic isoform of the prion protein and that
are kinetically favoured in binding to the beads. The
affinity to PrP^{27-30} could then be understood assuming that
native PrP^{Sc} present in sick mice is saturated with PrP^{B}
20   which might be released upon proteolytic digest.
Alternatively, partial proteolysis may expose PrP^{B}
binding sites on PrP^{Sc}. However, the fact that the
addition of PK-treated brain homogenate allows to bind
PrP^{Sc} indicates that there might be several different
25   interactions leading to our observations.

            The template-directed refolding hypothesis
predicts that PrP^{C} and PrP^{Sc} form heterodimers during the
conversion process. Therefore we investigated whether
PrP^{B} is identical with PrP^{C}. However, when coupling in
30   excess PrP^{B} activity was present in the serum of $Prnp^{o/o}$
mice at levels similar to those of wild-type mice,
implying that PrP^{C} does not contribute to the binding
activity (see Figure 8).

            If PrP^{B} activity is not only caused by the
35   special coupling conditions, it should be possible to
"purify" it by fractionating mouse serum by differential
ammonium sulfate precipitation. Indeed, it was possible

to precipitate PrP^B at an ammonium sulfate saturation
below 50% whereby coupling of each fraction was performed
in the presence of an excess of protein (see Figure 9).
While purified rabbit immunoglobulins against total mouse
5 serum did not contain PrP^B (data not shown), they
efficiently bound PrP$^{27-30}$ upon preincubation with full
mouse serum (see Figure 10, lanes 1-3) or with proteins
precipitating between 25% and 50% ammonium sulfate
saturation (see Figure 10, lanes 4-6). Preincubation with
10 proteins precipitating between 75% and 100% ammonium
sulfate saturation did not lead to PrP^B activity (see
Figure 10, lanes 7-9). This finding is important as it
shows that the PrP^B activity is a property of one or more
serum proteins independent of the covalent crosslink to
15 the surface of the beads.

         As the ammonium sulfate fractionation worked
with human serum as well (data not shown), 58 fractions
of human plasma were obtained by chromatography and
differential precipitation and tested for binding
20 activity to form an idea of the identity of PrP^B. All
fractions were not coupled in the presence of an excess
of proteins. Therefore the results can directly be
compared with 6H4 or mouse IgG. 20 fractions tested
positive: Plasminogen, fibrinogen, antithrombin III,
25 antithrombin III heparin complex, C1 esterase inhibitor,
factor IX and several fractions containing protein
mixtures (see Figure 11). Purified plasminogen and also
purified fibrinogen bound PrP^{Sc} in addition to PrP$^{27-30}$ (see
Figure 12). Out of the 38 fractions that tested negative,
30 6 contained purified proteins: Prothrombin complex
concentrate, albumin, activated prothrombin complex
concentrate, factor XIII and thrombin.

         As mentioned, there are some hints that the
binding of PrP$^{27-30}$ is caused by different effects. The
35 activity that binds PrP$^{27-30}$ is termed spPrP^B (s=serum and
p=plasma) as it is present in serum and in plasma. Said
activity is comparable to the activity found for

plasminogen and fibrinogen. Plasminogen and fibrinogen
were furthermore characterized as they both bind also
PrP^{Sc}.

As calcium is an important cofactor in the
5 coagulation cascade it was investigated whether PrP^B
activity is still intact if coagulation is inhibited by
complexing calcium. In the presence of 10 mM EDTA the
pathogenic PrP^{Sc} and PrP$^{27-30}$ were still bound by
plasminogen (see Figure 13, lanes 1-3) but only PrP$^{27-30}$ by
10 fibrinogen (see Figure 13, lanes 4-6). At least in the
case of plasminogen this finding speaks against the
possibility that the PrP^B activity is due to unspecific
coagulation. Because PrP^B selectively interacts with the
pathogenic PrP but not with PrP^C, interaction may be
15 conformation-specific. When the assay was carried out in
the presence of 6M urea the fraction containing purified
plasminogen didn't bind PrP^{Sc} nor PrP$^{27-30}$ (see Figure 14,
lanes 8-9) under these conditions PrP^{Sc} becomes protease-
sensitive (see Figure 14, lanes 14-15). As the
20 conformation of PrP^{Sc} is thought to be responsible for the
PK resistancy we conclude from this experiment that the
interaction of plasminogen and PrP^{Sc} is conformation-
dependant.

Furthermore it could be shown that PrP^B
25 activity of plasminogen is not dependent on the covalent
crosslink to the beads by using magnetic beads coated
with antibodies directed against plasminogen and
preincubated with plasminogen (see Figure 15, lanes 3-4).
There are two negative controls: 1. If beads coated with
30 antibodies against plasminogen are not at all
preincubated (see Figure 15, lanes 1-2) or preicubated
with albumin (see Figure 15, lanes 5-6), the pathogenic
isoform of PrP is not bound. 2. If beads coated with
albumin are preincubated with plasminogen there is also
35 no binding to the pathogenic isoform of PrP (see Figure
15, lanes 7-8).

Furthermore it could be shown that at least spPrP[B] does not only bind the pathogenic PrP but also infectivity. For this purpose we inoculated indicator tga20 mice i.c. with 0.2% of the paramagnetic beads
5   before eluting the other 99% of the beads and performing a western blot (see Figure 16). The animals that were inoculated with beads that bind the pathogenic PrP did all develop the disease (see Figure 17, lanes 4,5 and 7).

10

Examples:

Example 1: IAP method

The IAP protocol is the following: Bring the
15   brain tissue in a 15 ml FALCON tube, put it on ice and leave it there for all steps. Add Homogenate Buffer (0.5% DOC / 0.5% NP-40 in PBS) to get 10 % (w/v) homogenate. Pass the tissue through a 18 gauge needle and a 22 gauge needle by sucking up and down for 15 times each.
20   Centrifuge the homogenate for 30 minutes at 500 g and 4°C. Keep the supernatant. Determine the protein concentration. Centrifuge the homogenate for 30 minutes at 500 g and 4°C. Keep the supernatant. If the protein concentration is higher than 10 mg/ml then bring the
25   homogenate to a protein concentration of 10 mg/ml using the homogenate buffer. Bring the homogenate to a protein concentration of 5 mg/ml and 3% Tween 20 / 3% NP-40 all in PBS. Add to the tissue homogenate Proteinase K to get a final concentration of 50 μg/ml. Incubate for 60
30   minutes at 37°C. Add PMSF to get a final concentration of 5 mM. Add 0.25 volumes of IAP buffer (3% Tween 20 /3 % NP-40 in PBS). Resuspend the magnetic beads (covered with 6H4) according to the protocol described below) thoroughly. Pipette out 100 μl. Remove buffer. Add the
35   homogenate to the beads and incubate the bead-sample mixture with continous mixing for 1.5 hours at room temperature. Collect the beads using the MPC (strong

magnet). Wash three times with 1 ml Washing Buffer (2%
Tween 20 / 2% NP-40 in PBS) and once with 1 ml PBS by
vortexing for 15 seconds at room temperature and by using
the MPC. Spin down the beads, discard the remaining
5   supernatant using again the MPC. Add 24 µl x Loading
Buffer (50 mM Tris pH 6,8; 2% SDS; 0.01% bromphenol blue;
10% glycerol). Heat to 95°C for 5 minutes. If the samples
are stored at -20°C then heat them again for 30 seconds
at 95°C before performing SDS-PAGE followed by western
10   Blot: Assemble the glass plates according to the
manufacturer's instructions. Prepare in a Falcon tube the
appropriate volume of the Resolving Gel (2.1 ml H₂O, 1.5
ml 40 % Acrylamid, 1.3 ml 1.5 M Tris pH 8.8, 50 µl 10 %
SDS, 50 µl 10 % Ammoniumpersulfat, 2 µl TEMED). Mix the
15   components in the order shown. Polymerization will begin
as soon as the TEMED has been added. Pour the acrylamide
solution into the gap between the glass plates. Leave
sufficient space for the stacking gel (the length of the
comb plus 1 cm). Using a pasteur pipette carefully
20   overlay the acrylamide with water. Place the gel in a
vertical position at room temperature. After
polymerization is complete (30 minutes), pour off the
overlay and wash the top of the gel several times with
deionized water to remove any unpolymerized acrylamide.
25   Prepare in a Falcon tube the appropriate volume of the
Stacking Gel (1.48 ml H2O, 0.25 ml 40 % Acrylamid,
0.25 ml 1.0 M Tris pH 6.8, 20 µl 10 % SDS, 20 µl 10 %
Ammoniumpersulfat, 2 µl TEMED). Mix the components in the
order shown. Polymerization will begin as soon as the
30   TEMED has been added. Pour the stacking gel solution
directly onto the surface of the polymerized resolving
gel. Immediately insert a clean Teflon comb into the
stacking gel solution, being careful to avoid trapping
air bubbles. Place the gel in a vertical position at room
35   temperature. After polymerization is complete (30
minutes), remove the Teflon comb carefully. Mount the gel
in the electrophoresis apparatus. Add Running buffer to

the top and bottom reservoirs. Remove any (25 mM Tris,
250 mM glycine, 0.1 % SDS) bubbles that become trapped at
the bottom of the gel between the glass plates. Load 24
µl of each of the samples in a predetermined order into
5 the bottom of the wells (1. well: Low -range marker).
Load an equal volume of 1x Gel-loading Buffer into any
wells that are unused. Attach the electrophoresis
apparatus to an electric power supply (the positive
electrode should be connected to the bottom reservoir).
10 Apply 10 V/cm to the gel. After the dye front has moved
into the resolving gel (30 minutes), increase the voltage
to 14 V(cm and run the gel until the bromophenol blue
reaches the bottom of the resolving gel (1 hour). Then
turn off the power supply. Cut six sheets of absorbent
15 paper (Whatman 3MM or equivalent) and one sheet of
nitrocellulose to the size of the gel (6cm x 8 cm). If
the paper overlaps the edge of the gel, the current will
short-circuit the transfer and bypass the gel, preventing
efficient transfer. Wet the absorbent paper, the
20 nitrocellulose and the gel by soaking in Transfer (39 mM
glycine, 48 mM Tris, 0.037 % SDS, 20 % methanol) Buffer.
On the bottom plate of the apparatus (the anode),
assemble the gel, nitrocellulose, and paper in this
order:
25   bottom electrode,
        three layers absorbent paper soaked in
transfer buffer,
        one nitrocellulose membrane soaked in
transfer buffer,
30   polyacrylamide gel slightly wetted with
transfer buffer,
        three layers absorbent paper soaked in
transfer buffer.
        Check carefully for air bubbles and gently
35 remove them either by using a gloved hand or by rolling a
pipet over the sandwich. Dry any buffer that may surround
the gel-paper sandwich. Carefully place the upper

electrode (the cathode) on top of the stack. Put a weight
on it. Connect the electrodes and commence transfer.
Running time is 1 hour with a current of 1 mA/cm^2. After
transfer, disconnect the power source. Carefully
5　disassemble the apparatus. Mark membrane to follow
orientation (usually by snipping off lower left-hand
corner, the number one lane). Rinse the membrane three
times with TBS-T. Add Blocking Buffer (5 % (w/v) nonfat
dry milk in TBS-T). Incubate at room temperature with
10　agitation for 30 minutes. Rinse the membrane three times
with TBS-T. Add to 2.5 µl of mAB 6H4 (2 mg/ml) 12.5 ml of
1% (w/v) nonfat dry milk in TBS-T. Incubate at room
temperature with agitation for 1 hour or overnight at
4°C. Remove the membrane from the antibody solution and
15　wash three times for 10 minutes each in TBS-T. Add to
1.25 µl of relativ anti mouce IgG1-HRP 12.5 ml of 1%
(w/v) nonfat dry milk in TBS-T. Incubate at room
temperature with agitation for 1 hour. Remove the
membrane from the antibody solution and wash three times
20　for 15 minutes each in TBS-T. Mix 1 ml of detection
solution 1 with 1 ml of detection solution 2 from the ECL
Western blotting detection reagents (Amersham Pharmacia
Biotech). Incubate for precisely 1 minute at room
temperature without agitation. Drain off excess detection
25　reagent by putting the membrane on a absorbent paper.
Gently place the membrane, protein side down, on a
SaranWrap. Close SaranWrap to form a envelope avoiding
pressure on the membrane. Place the membrane, protein
side up, in the film cassette. Work as quickly as
30　possible. Switch off the lights and carefully place a
sheet of autoradiography film such as (Hyperfilm ECL) on
top of the membrane, close the cassette and expose for
some seconds (15", 30").

35

Example 2: PAA method

Couple the protein of interest to magnetic
beads: Bring 100 µg of protein into approx. 1ml of
Coupling Buffer (0.1 M borate buffer pH 9.5: dissolve
6.183 g H3BO3 in 800 ml distilled water, Adjust pH to 9.5

5   using 5 M NaOH and adjust volume to 1000 ml with
distilled water; if necessary, change buffer by
dialysis). (If coupling was performed in the presence of
an excess, 1 mg was used for 1 ml of coupling buffer.)
Make a homogeneous suspension of the Dynabeads M-280

10 Tosylactivated by Dynal using a pipette and by vortexing
for approximately 1 min. Pipette out 1 ml of Dynabeads
and wash as follows: Place the tube in the DYNAL MPC.
Leave to separate for 2 minutes. Remove the supernatant
taking care not to disturb the Dynabeads. Remove the tube

15 from the Dynal MPC and resuspend the Dynabeads in PBS.
Repeat these steps and resuspend the Dynabeads in the
coupling buffer containing the antibodies. Incubate for
24 h at 37°C with tilt rotation. Place the tube in the
magnet for 3 minutes and remove the supernatant. Wash the

20 coated Dynabeads six times: 2 x in PBS/BSA (add 0.1 %
(w/v) bovine serum albumin (final concentration) to PBS),
pH 7.4 for 5 minutes at room temperature; 1 x in Blocking
Buffer (0.2 M Tris pH 8.5 with 0.1 % (w/v) BSA: dissolve
2.42 g Tris in 80 ml distilled water. Adjust pH to 8.5

25 using 1 M HCl, add 0.1 % BSA and adjust volume to 100 ml
with distilled water) for 4 h at 37°C; 1 x in PBS/BSA, pH
7.4 for 5 minutes at room temperature; 1 x in 1% Tween 20
for 10 minutes;1 x in PBS/BSA, pH 7.4 for 5 minutes at
room temperature. Store the coated Dynabeads in PBS/BSA

30 pH 7.4, 0.02% sodium azide. Then prepare Sample I: Add 1
ml of PAA Buffer (3 % NP-40 / 3 % Tween 20 in PBS) to 10
µl of not infected brain homogenate (Protein
concentration 5 mg/ml; 0.5% DOC / 0.5 NP-40). Then
prepare Sample II and III: Add 1 ml of PAA Buffer (3 %

35 NP-40 / 3 % Tween 20 in PBS) to 10 µl of infected brain
homogenate (Protein concentration 5 mg/ml; 0.5% DOC / 0.5
NP-40). Incubate Sample I and Sample II for 30 minutes at

37°C without PK. Incubate Sample III for 30 minutes at
37°C with PK at final concentration of 50 µg/ml (add 50
µl of PK 1mg/ml). Add PMSF to all samples to get a final
concentration of 5 mM (add 50 µl of 100 mM PMSF).

5   Resuspend the Magnetic Beads thoroughly. Pipette out 100
µl. Add the beads to the Samples and incubate the bead-
sample mixture with continuous mixing for 1.5 hours at
room temperature. Collect the beads using the MPC. Wash
three times with 1 ml Washing Buffer and once with 1 ml

10 PBS by vortexing for 15 seconds at room temperature and
by using the MPC. Spin down the beads, discard the
remaining supernatant using again the MPC. Add 24 µl 1 x
Loading Buffer. Heat to 95°C for 5 minutes. If the
samples are stored at -20°C then heat them again for 30

15 seconds at 95°C before loading on the gel.

As a positive control of this assay 6H4 is
used and as a negative control mouse IgG or mouse albumin
(see Figure 4).

20   Example 3

In order to investigate whether a given mouse
serum containes IgG that specifically recognize PrP^{Sc}
magnetic beads that are already coated by the company
DYNAL with sheep antibodies directed against mouse IgGs

25 were used after preincubation with mouse serum. These
beads were the first negative control. As a second
negative control these beads preincubated with normal
mouse serum were used in order to show that IgGs from
normal mouse serum do not bind to any form of PrP.

30 Surprisingly the beads alone showed an affinity to PrP^{Sc}
but not to PrP$^{27-30}$. Upon preincubation with normal mouse
serum also PrP$^{27-30}$ is bound (see Figure 5). Therefore it
was hypothised that the sheep antibodies from DYNAL
recognize a molecule that is associated with PrP^{Sc} but

35 digested away after PK-treatment. As PrP$^{27-30}$ is bound upon
preincubation with normal mouse serum, this serum might
contain the molecule with affinity to PrP^{Sc}.

Example 4

The beads coupled to total mouse serum
proteins did not show any affinity to any form of PrP.
However, if the coupling of the total serum was performed
5 in the presence of an excess of protein the beads showed
the same binding to PrP^{27-30} as the monoclonal antibody 6H4
whereas the beads that were coupled in the presence of an
excess of albumine still did not show any affinity to any
form of PrP (see Figure 6). Though it was not possible to
10 measure any difference of the coupling efficency of the
two conditions it might be that offering an excess of
proteins causes a sponge on the surface of the beads that
binds PrP^{27-30}.

15   Example 5

We also checkled whether PK-treated brain
homogenate might enhance the binding as in the case of
bound PrP^{27-30} total PK-digested brain homogenate is
present: the addition of PK-digested brain homogenate
20 from wild-type C57BL/6 mice or $Prnp^{o/o}$ mice allowed to
bind PrP^{Sc} in addition to PrP^{27-30}; the addition of
inactive PK had no influence on the binding activity (see
Figure 7).

25   Example 6

If coupled in the presence of an excess the
activity of binding PrP^{27-30} was also found in the serum of
man, sheep, cow and in the serum of terminally scrapie-
sick C57BL/6 mice (data not shown).

30

Example 7

The template-directed refolding hypothesis
predicts that PrP^{C} and PrP^{Sc} form heterodimers during the
35 conversion process. Therefore we investigated whether
PrP^{B} is identical with PrP^{C}. However, when coupling in
excess PrP^{B} activity was present in the serum of $Prnp^{o/o}$

mice at levels similar to those of wild-type mice,
implying that PrP^C does not contribute to the binding
activity (se Figure 8).

5   Example 8

If PrP^B activity is not only caused by the
special coupling conditions, it should be possible to
"purify" it by fractionating mouse serum by differential
ammonium sulfate precipitation. Indeed, it was possible
10 to precipitate PrP^B at an ammonium sulfate saturation
below 50% whereby coupling of each fraction was performed
in the presence of an excess of protein (see Figure 9).
While purified rabbit immunoglobulins against total mouse
serum did not contain PrP^B (data not shown), they
15 efficiently bound PrP$^{27-30}$ upon preincubation with full
mouse serum or with proteins precipitating between 25%
and 50% ammonium sulfate saturation. Preincubation with
proteins precipitating between 75% and 100% ammonium
sulfate saturation did not lead to PrP^B activity (see
20 Figure 10). This finding is important as it shows that
the PrP^B activity is a property of one or more serum
proteins independent of the covalent crosslink to the
surface of the beads.

25   Example 9

As the ammonium sulfate fractionation worked
with human serum as well (data not shown), 58 fractions
of human plasma were obtained by chromatography and
differential precipitation and tested for binding
30 activity to form an idea of the identity of PrP^B. All
fractions were not coupled in the presence of an excess
of proteins. Therefore the results can directly be
compared with 6H4 or mouse IgG. 20 fractions tested
positive: Plasminogen, fibrinogen, antithrombin III,
35 antithrombin III heparin complex, C1 esterase inhibitor,
factor IX and several fractions containing protein
mixtures (see Figure 11). Purified plasminogen and also

purified fibrinogen bound PrP^{Sc} in addition to PrP$^{27-30}$ (see
Figure 12). Out of the 38 fractions that tested negative,
6 contained purified proteins: Prothrombin complex
concentrate, albumin, activated prothrombin complex
5 concentrate, factor XIII and thrombin.

Example 10

As calcium is an important cofactor in the
coagulation cascade it was investigated whether PrP^{B}
10 activity is still intact if coagulation is inhibited by
complexing calcium. In the presence of 10 mM EDTA the
pathogenic PrP^{Sc} and PrP$^{27-30}$ were still bound by
plasminogen but only PrP$^{27-30}$ by fibrinogen (Fig. 13). At
least in the case of plasminogen this finding speaks
15 against the possibility that the PrP^{B} activity is due to
unspecific coagulation.

Example 11

Because PrP^{B} selectively interacts with the
20 pathogenic PrP but not with PrP^{C}, interaction may be
conformation-specific. When the assay was carried out in
the presence of 6M urea the fraction containing purified
plasminogen didn't bind PrP^{Sc} nor PrP$^{27-30}$; under these
conditions PrP^{Sc} becomes protease-sensitive (Fig. 14). As
25 the conformation of PrP^{Sc} is thought to be responsible for
the PK resistancy we conclude from this experiment that
the interaction of plasminogen and PrP^{Sc} is conformation-
dependant.

30   ### Example 12

Furthermore it could be shown that PrP^{B}
activity of plasminogen is not dependent on the covalent
crosslink to the beads by using magnetic beads coated
with antibodies directed against plasminogen and
35 preincubated with plasminogen (Fig. 15).

Example 13

Furthermore it could be shown that at least spPrP^B does not only bind the pathogenic PrP but also infectivity. For this purpose we inoculated indicator
5   tga20 mice i.c. with 0.2% of the paramagnetic beads before eluting and performing a western blot. The animals that were inoculated with beads that bind the pathogenic PrP did all develop the disease (Fig. 16, Fig. 17).

While there are shown and described presently
10 preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

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Claims

1. A method for the concentration of PrP^{Sc} or digestion products thereof, wherein a body fluid or
5 fluidized organ is treated with solid phase material such as e.g. magnetic beads (MB) whereby at least part of said material or beads, respectively, carries a prion binding site.

2. The method of claim 1, wherein the prion
10 binding site is a factor with prion binding activity (PrPB).

3. The method of claim 1, wherein the fluidized organ is homogenized tissue of central nervous system.

15 4. The method of claim 1, wherein the fluidized organ is homogenized brain tissue.

5. The method of claim 1, wherein the fluid is a fluid that has been digested by proteinase K (PK).

6. The method of claim 2, wherein the solid
20 phase materials, e.g. MBs, carrying PrPB are prepared by coupling MBs with blood serum, or blood plasma.

7. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling solid phase material with serum or plasma
25 fraction II of ammonium sulfate precipitation.

8. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling MBs with material selected from the group consisting of plasma fraction I of ammonium sulfate
30 precipitation, plasminogen and fibrinogen.

9. The method of claim 1, wherein the solid phase materials, e.g. MBs, carrying PrPB are prepared by coupling solid phase material with plasminogen, fibrinogen or sPrPBII or pPrPBII.

35 10. A method for the detection, and optionally quantification, of PrP^{Sc} or digestion products thereof, wherein PrP^{Sc} is first concentrated according to

one of claims 1 to 6 and then detected, and optionally compared with a standard.

11. The method of claim 10, wherein the detection is performed by Western blot analysis.

12. sPrPBII which is a factor with prion binding activity in fraction II of ammonium sulfate precipitation of serum.

13. pPrPBII which is a factor with prion binding activity in fraction II of ammonium sulfate precipitation of normal or fresh frozen plasma.

14. spPrPB which is a factor with prion binding activity in total serum or plasma.

15. Solid phase material, such as MBs, carrying a PrPB.

16. A composition for the purification of body fluids and/or the sterilization of surgical or diagnostic tools comprising a PrPB, preferably coupled to a solid phase material.

17. A method for the sterilization of surgical or diagnostic tools wherein said tools are treated with a PrPB comprising composition, preferably a composition comprising PrPB coupled to a solid phase material.

18. A method for the concentration and/or isolation of PrPBs wherein serum or plasma is subjected to fractionated ammonium sulfate precipitation thus that at least one PrPB of interest is precipitated, preferably in only one fraction.

19. The method of claim 18, wherein the PrPB comprising fraction is further purified by further protein isolation methods.

20. A method for purification and/or removal of pathological prion protein from body fluids or fluidized organs, such as blood, urine, cerebrospinal fluid, brain tissue, lymph nodes, tonsils, or for the sterilization of surgical and/or diagnostic tools basing

on the affinity of PrPB for the pathological prion
protein.

           21. A method for the purification of body
fluids, e.g. blood units, wherein the fluid is treated
with plasminogen.

           22. A therapy regimen based on the modulation
of production of PrPB for preventing the spread of prions
in the body.

           23. The regimen of claim 22, wherein the PrPB
is plasminogen.

           24. A test for the detection of pathological
prion protein in body fluids or organs such as blood,
urine, cerebrospinal fluid, brain tissue, lymph nodes,
tonsils etc, that utilizes the specific binding
properties of PrPB to pathological prion protein.

           25. The test of claim 24 that is embodied as
a microtiter plate format immunoassay, e.g. ELISA assay,
an immunoprecipitation assay, a BIACORE assay, a
immunocytochemical assay, a histoblot assay, etc.

           26. A DNA sequence specific for biosynthesis
of PrPB and/or an expression vector comprising same.

           27. A method for purification of PrPB by
using PrP^{27-30} as bait.

           28. Monoclonal and polyclonal antibodies
produced in animals, such as mice, rabbits, chicken, or
other species, and directed against PrPB.

           29. Single-chain Fv fragments and other types
of fragments of antibodies produced in recombinant phages
or in other recombinant systems, and directed against
PrPB.

           30. A test predictive of susceptibility to
prion diseases based on polymorphisms of PrPB, or on
variations in the strength and pattern of production of
PrPB.

           31. A transgenic animal, in particular mouse
that overproduces PrPB in brain, lymph nodes, or other
organs, to be used in a bioassay for prions.

32. A knockout animal, in particular a
knockout mouse, which is devoid of PrPB, to be used in a
bioassay for prions.

33. Production method of PrPB by expressing a
5   DNA sequence specific for the biosynthesis of PrPB in a
suitable host cell, such as bacteria, yeast, fungi, or
eukaryotic cells, and by purification of PrPB from the
aforementioned organisms.

34. Use of natural or synthetic PrPB as a
10   medicament for therapeutical applications in humans and
animals.

35. A vaccination of organisms with natural
or synthetic PrPB, in particular PrPBIp.

36. A diagnostic assay for human and/or
15   animal diseases resulting from abnormal production and/or
metabolism of PrPB.

Fig. 1

Fig. 2

Fig. 3

● Magnetic Beads coated with the protein of interest

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Fig. 4

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Fig. 5

6/17

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fraction	PrPc	PrPc	PrPsc	PrPsc PrP27-30
Plasminogen	-		+	+
Fibrinogen	-		+	+
Antithrombin III	-	-		+
C1 esterase inhibitor	-	-		+
Antithrombin III heparin complex	-	-		+
Plasma pool	-	-		(+)
Factor IX, Fibrin sealant 2 (!)	-	-		+
12 fractions containing protein mixtures				
Total: 20 positive fractions				
Prothrombin complex concentrate	-	-	-	
Human albumin	-	-	-	
Activated prothrombin complex concentrate	-	-	-	
Factor VIII concentrate	-	-	-	
Human Thrombin	-	-	-	
Factor XIII	-	-	-	
Factor IX concentrate, Fibrin sealant 1 (!)	-	-	-	
30 fractions containing protein mixtures				
Total: 38 negative fractions				

Fig. 11

Fig. 12

Fig. 13

Fig. 14

Fig. 15

Western Blot

i.c. inoculation of
tg20 mice

90%

0.2%

Washing

PrPC, PrPSc
and PrP27-30

● Magnetic Beads coated with the
protein of interest

Fig. 16

Fig. 17

	1	2	3	4	5	6	7
Incubation Time (d)	>110	>110	>110	82 89	71 76	>110	72 82
	>110	>110	>110	91 93	76 84	>110	82 87
	>110	>110	>110	93	84	>110	91
	>110	>110	>110			>110	
	>110	>110	>110			>110	
Mean (d)	>110	>110	>110	89.6	78.2	>110	82.8
STDV (d)				4.6	5.7		7.1
log LD_{50} (on total beads)	<2.9	<2.9	<2.9	4.7	5.7	<2.9	5.3
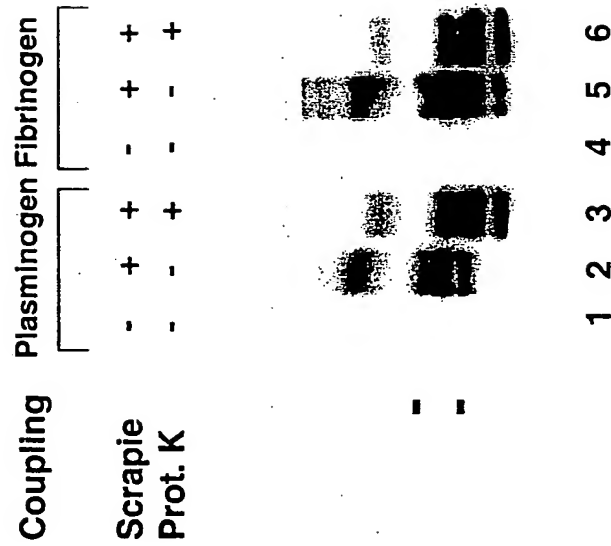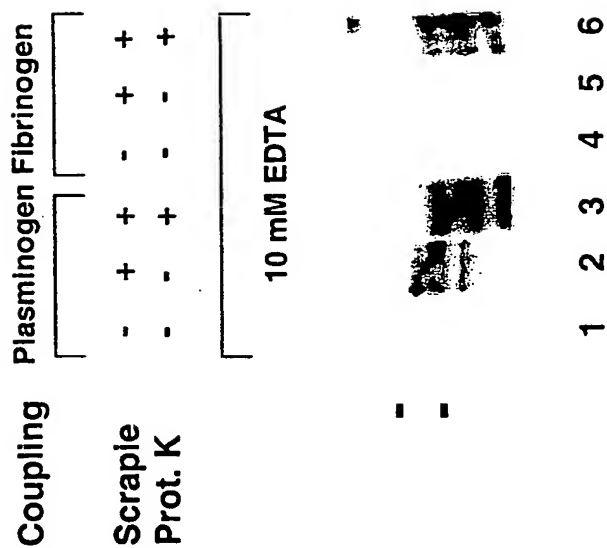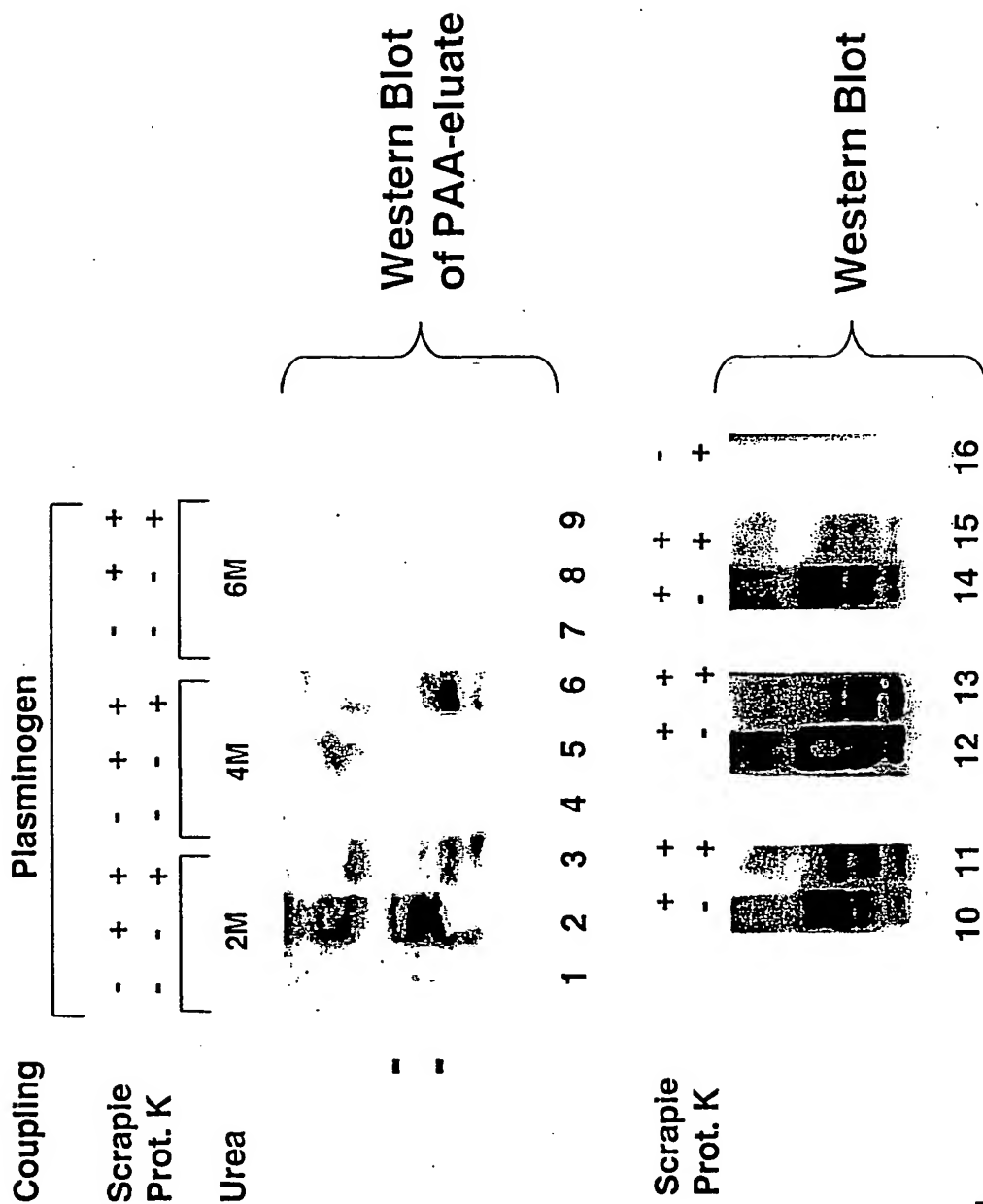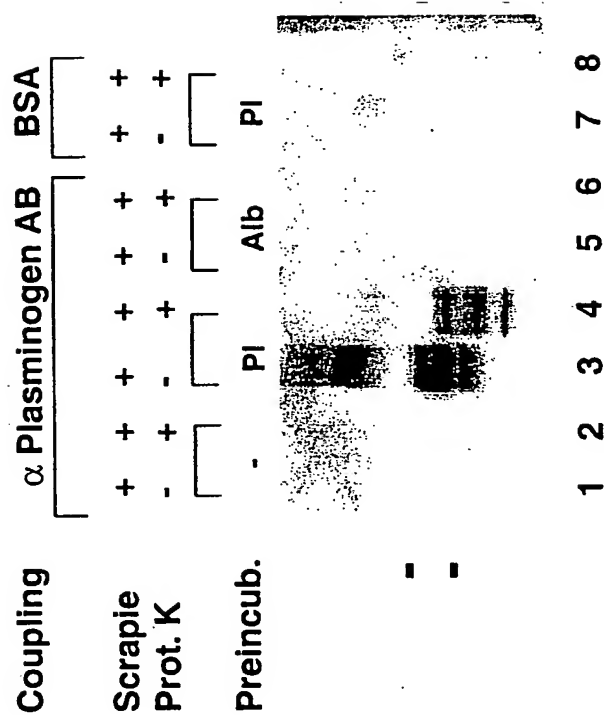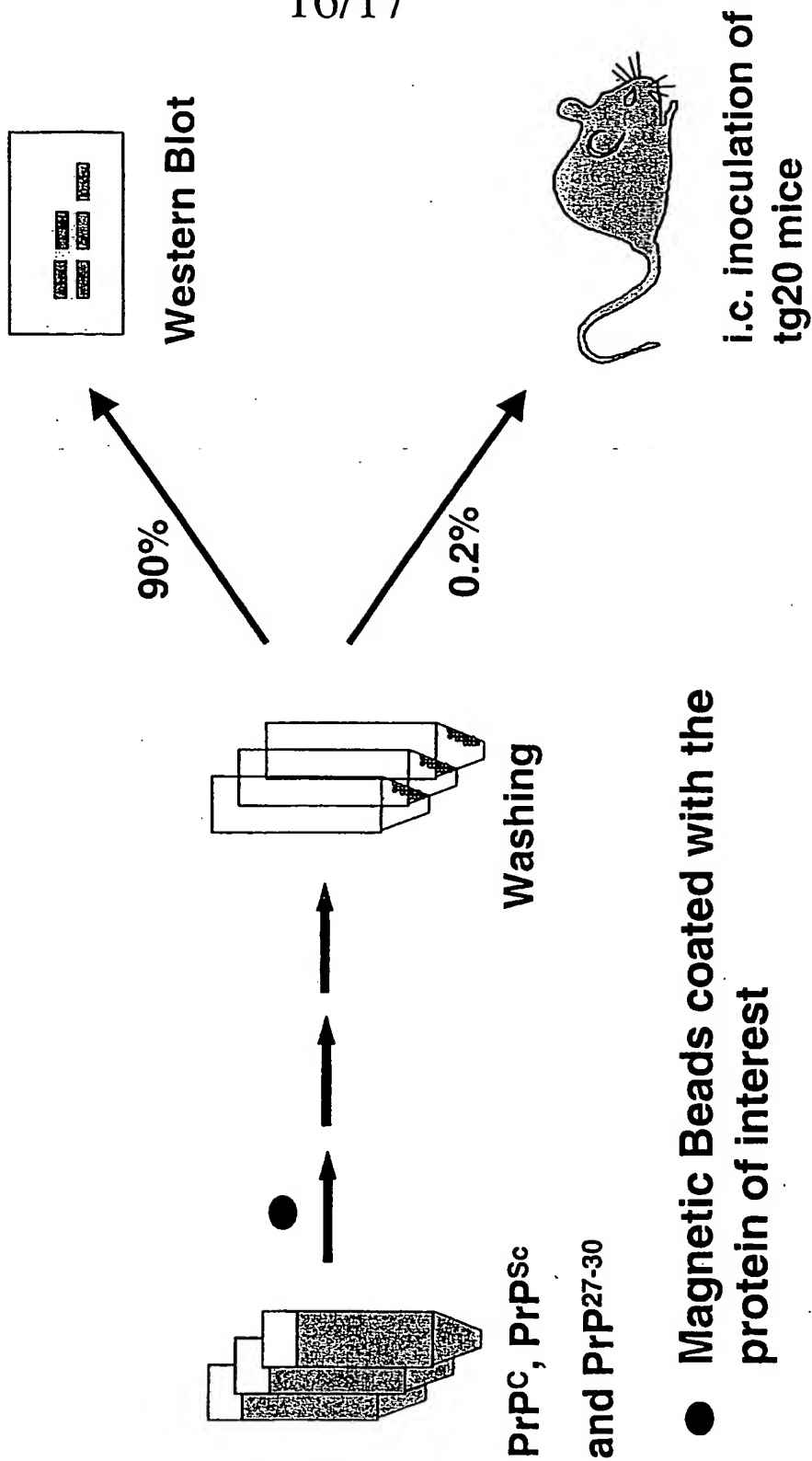
Coupling: MSA, 6H4, NMS*

Scrapie / Prot. K

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C07K14/47   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C07K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 846 533 A (BURTON DENNIS R ET AL) 8 December 1998 (1998-12-08) column 9, line 61, claims ---	1-11, 15-30, 33-36
X	WO 97 43649 A (WINNACKER ERNST LUDWIG ;WEISS STEFAN (DE); EDENHOFER FRANK (DE); R) 20 November 1997 (1997-11-20) claims, examples, page 6, second part "bound to solid phase" ---	1-11, 15-30, 33-36
P,X	WO 00 26238 A (IMP COLLEGE INNOVATIONS LTD ;JACKSON GRAHAM STUART (GB); COLLINGE) 11 May 2000 (2000-05-11) examples, esp. page 68, item 23, item 7, page 32ff ---	1-30, 33-36

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2000	1 0. 10. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Kronester-Frei, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LIU, AIZHUO ET AL: "NMR experiments for resonance assignments of 13C, 15N doubly-labeled flexible polypeptides: . Application to the human prion protein hPrP(23-230" JOURNAL OF BIOMOLECULAR NMR, (FEB., 2000) VOL. 16, NO. 2, PP. 127-138. , XP000918003 whole document, esp. Methods on page 128ff	1-30, 33-36
P,Y	SWIETNICKI W ET AL: "Aggregation and fibrillization of the recombinant human prion protein huPrP90 -231." BIOCHEMISTRY, (2000 JAN 18) 39 (2) 424-31. , XP000887071 Abstract, page 425, left column	1-30, 33-36
E	WO 00 43782 A (SAFAR JIRI G`;UNIV CALIFORNIA (US); PRUSINER STANLEY B (US)) 27 July 2000 (2000-07-27) whole document, esp. page 22	1-30, 33-36
Y	JACKSON G S ET AL: "REVERSIBLE CONVERSION OF MONOMERIC HUMAN PRION PROTEIN BETWEEN NATIVE AND FIBRILOGENIC CONFORMATIONS" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 283, no. 283, 1999, pages 1935-1937, XP000887243 ISSN: 0036-8075 whole document, esp. page 1935 right column, Fig 3, digestion with PK	1-30, 33-36
Y	SHAKED G M ET AL: "Protease-resistant and detergent-insoluble prion protein is not necessarily associated with prion infectivity." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 JUN 18) 274 (25) 17981-6. , XP000919442 Abstract	1-30, 33-36
Y	AUCOUTURIER, PIERRE (1) ET AL: "Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt-Jakob disease." SOCIETY FOR NEUROSCIENCE ABSTRACTS., (1999) VOL. 25, NO. 1-2, PP. 1854. MEETING INFO.: 29TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE. MIAMI BEACH, FLORIDA, USA OCTOBER 23-28, 1999 SOCIETY FOR NEUROSCIENCE. , XP000917988 Abstract, Fig. 1	1-30, 33-36

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	RISOEN P A ET AL: "One-step magnetic purification of recombinant DNA-binding proteins using magnetizable phosphocellulose." PROTEIN EXPRESSION AND PURIFICATION, (1995 JUN) 6 (3) 272-7. , XP000917983 whole document, esp. page 273, right column	1-30, 33-36
Y	EP 0 332 022 A (BEHRINGWERKE AG) 13 September 1989 (1989-09-13) claims, page 4, lines 25ff	1-30, 33-36
A	EP 0 861 900 A (ZUERICH ERZIEHUNGSDIREKTION) 2 September 1998 (1998-09-02) claims, examples	1-30, 33-36
A	SCHREUDER B E C ET AL: "PRECLINICAL TEST FOR PRION DISEASES" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 381, 13 June 1996 (1996-06-13), page 563 XP000579899 ISSN: 0028-0836 whole document	1-30, 33-36

2

INTERNATIONAL SEARCH REPORT

International Application No.
PCT 0/00849

Box I   Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   31,32
   because they relate to subject matter not required to be searched by this Authority, namely:

   see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of Invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.1

Although claims 24, 30 and 36 are directed to a diagnostic method
practised on the human/animal body, although in claims 1-21 the
possibiliy of an in vivo method has not been explicitly excluded, the
search has been carried out and based on the alleged effects of the
compound/composition.
Although claim 34 is directed to a method of treatment of the
human/animal body, the search has been carried out and based on the
alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 31,32

Rule 39.1(ii) PCT - Transgenic animal or knockout animal (animal
variety) per se used in bioassay

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB ███49

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5846533	A	08-12-1998	AU 707484 B		08-07-1999
			AU 7073596 A		01-04-1997
			CA 2231409 A		20-03-1997
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